Journal of Surgical Oncology 1999;72:225–229

Expression of Receptors for Estrogen and Progesterone in Malignant Colonic Mucosa as a Prognostic Factor for Patient Survival

IOANNIS G. KAKLAMANOS, MD,[1]* OLIVER F. BATHE, MD,[1] DIDO FRANCESCHI, MD,[1] ANDREAS CH. LAZARIS, MD,[2] PANAGIOTIS DAVARIS, MD,[2] MIKES GLINATSIS, MD,[2] AND BASIL CH. GOLEMATIS, MD[2]

[1]*Division of Surgical Oncology, Sylvester Comprehensive Cancer Center, University of Miami School of Medicine, Miami, Florida*

[2]*The Athens University Medical School, Athens, Greece*

Background and Objectives: Estrogen receptors (ER) and progesterone receptors (PR) have been detected in both normal and malignant colonic mucosa, but the prognostic value of this observation is unknown. We aimed to define the prognostic significance of the presence of ER and PR in malignant cells from colorectal adenocarcinoma specimens.

Methods: An immunohistochemical assay for ER and PR was performed on paraffinized sections from 65 colorectal adenocarcinoma specimens. Survival curves were analyzed to define the prognostic implications of ER and PR.

Results: Twenty nine (45%) tumors tested receptor positive (32% for ER and 23% for PR). Tumors of advanced stage were more likely to express receptors than early stage tumors (56% vs. 32%; $P = 0.01$). Median survival of patients with neoplasms expressing PR was 30 months. For patients whose tumors did not express any receptors, median survival had not been reached at the time of follow-up ($P = 0.04$). Similarly, patients with tumors expressing both receptors had significantly reduced survival (median survival = 20 months; $P = 0.003$).

Conclusions: Expression of receptors for sex steroids correlates with advanced stage disease. Expression of PR by the tumor cells is associated with a shorter patient survival. The results suggest that sex steroids may play a role in carcinogenesis and tumor progression.

J. Surg. Oncol. 1999;72:225–229.

KEY WORDS: **colorectal cancer; adenocarcinoma; steroids; immunohistochemistry; receptors; prognosis**

INTRODUCTION

The importance of estrogen receptors (ER) and progesterone receptors (PR) in malignant neoplasms arising from tissues in which normal growth and function are largely regulated by sex steroid hormones has been recognized in the past [1]. For neoplasms of the breast, endometrium, ovary, and prostate, the significance of sex steroid hormones is indisputable and hormonal manipulation is part of their treatment [2–4].

For gastrointestinal neoplasms, there is only indirect evidence that steroid hormones may play a role in the development and progression of neoplasias. Although epidemiologic studies have shown an inverse association between the use of hormone replacement therapy and the incidence of colon cancer in postmenopausal women

*Correspondence to: Ioannis G. Kaklamanos, MD, PhD, University of Miami School of Medicine, Sylvester Comprehensive Cancer Center, Division of Surgical Oncology (310T), 1475 NW 12th Avenue, Room 3550, Miami, FL 33136. Fax No.: (305) 243-4907.
E-mail: ikaklama@mednet.med.miami.edu

Accepted 26 August 1999

[5,6], there is an epidemiologic association of colon cancer with cancer of the breast [7,8]. Previous studies have shown the presence of ER and PR in the normal epithelium of the gastrointestinal tract in some individuals [9–11]. Expression of ER and PR in adenocarcinoma specimens of the esophagus, stomach, gallbladder, pancreas, and colon has also been shown [12–16], but the significance of this observation is unknown. There is evidence that the presence of ER in malignant cells of the gastric mucosa is an independent negative prognostic factor for survival in patients with adenocarcinoma of the stomach [17,18]. However, the prognostic significance of ER and PR for cancer of the colon and rectum has not been well studied.

In this study, we examined pathology specimens from patients who underwent resection for colorectal cancer to determine expression of ER and PR. Our objectives were to correlate these findings with other clinicopathologic variables and with long-term outcome.

MATERIALS AND METHODS

We retrospectively studied paraffinized tumor specimens from 65 patients (35 men, 30 women) with adenocarcinoma of the colon and rectum. The mean age of the group was 67 ± 12 years (range 18–86 years). Only 2 of the 30 female patients were premenopausal and none of the postmenopausal women was receiving estrogen replacement therapy at the time of the diagnosis. Patients received adjuvant chemotherapy after curative resection, as indicated by the stage of the disease. Adenocarcinomas were staged according to the guidelines of the American Joint Committee on Cancer [19]. Twenty of 36 patients with rectal cancer received either postoperative or preoperative radiation therapy. Mean length of follow-up in patients still alive at the time of analysis was 60 months.

Specimen Processing

The paraffinized specimens were dewaxed by incubation at 60°C for 20 min, followed by two 20-min baths in xylene. Rehydration was achieved by processing the specimens through graded alcohol (100%, 96%, and 80% respectively), followed by immersion in distilled water for 3–5 min.

Assay for ER and PR

We used the monoclonal mouse anti-human ER antibody (clone 1D5, isotype IgG1, kappa; Dako, Glostrup, Denmark) and the Abbott PR-ICA kit (Abbott Labs, Abbott Park, IL) for determination of ER and PR, respectively. The tissue slides were washed twice with phosphate-buffered saline (PBS), then incubated in a solution containing PBS and 3% H_2O, for blocking endogenous peroxidase activity. Three more washes with PBS followed. To optimize antigen retrieval, the slides were superheated in 0.01 M citrate buffer (pH 6.0) according to the technique of "pressure heating" described by Norton et al. [20].

Swan serum in PBS was used as a blocking reagent. After incubation at room temperature for 30 min and removal of the excess blocking reagent, the primary antibody (murine monoclonal IgG to human ER and PR) was added, then incubated for 45 min at room temperature. The slides were again washed with PBS. As a second antibody, for detection of ER we used biotinylated rabbit anti-mouse IgG (Dako) at a concentration of 1.3 g/L in 0.01 M PBS and 15 μM NaN_3 (pH 7.4). Then sections were incubated with ABC-streptavidin (Dako). Diaminobenzidine (DAB-HCl) was added as chromogen substrate and the slides were incubated for 45 min.

All the stained tissue slides were examined under the light microscope by the same examiner who was blind to the clinical data. A semiquantitative grading system was used. Staining was considered positive when at least 15% of the cells stained positive for ER or PR. We used a slide processed without the addition of the primary antibody as a negative control. Normal endometrial tissue was used as a positive control.

Data Analysis

Expression of receptors between different groups was compared with the chi-square test. Survival curves were calculated by the Kaplan-Meier method and compared by the log-rank test. Multivariate analysis was performed using the Cox proportional hazards model. The statistical analysis was performed using SPSS TM version 8.0 (SPSS Inc., Chicago, IL). Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

In immunopositive tumors, the immunostain localized in the nucleus of 15–75% of malignant cells. Twenty-nine (45%) tumor specimens tested positive for receptors. Twenty-one (32%) specimens were ER positive, 15 (23%) were PR positive, and 7 (10%) specimens were positive for both receptors. Women were more likely to express PR than men ($P < 0.01$). In patients with advanced disease (i.e., stages III, IV), the incidence of receptors in malignant tissue was higher than in those with early disease (i.e., stages I, II) (32% vs. 56%; $P < 0.05$). This difference was even more pronounced for PR (6% vs. 38%; $P = 0.01$). The receptor status according to gender, stage, tumor differentiation, and tumor location is summarized in Table I. There was no significant correlation between the degree of differentiation and the presence of receptors.

The overall 5-year survival rate was 55%. Median survival had not been reached at the time of follow-up for receptor negative patients. Patients with receptor positive tumors had a median survival of 45 months and a 5-year

TABLE I. ER and PR Receptor Status in Patients with Adenocarcinoma of the Colon and Rectum, According to Tumor and Patient Characteristics

	ER	PR	ER + PR	Negative	Total
Gender					
Male	12	3	2	22	35
Female	9	12	5	14	30
Tumor location					
Colon	8	10	5	16	29
Rectum	13	5	2	20	36
Degree of differentiation					
Well differentiated	3	3	1	8	13
Moderately differentiated	14	8	3	20	39
Poorly differentiated	4	4	3	8	13
Stage					
I	1	2	1	3	5
II	8	0	0	18	26
III	8	8	3	13	26
IV	4	5	3	2	8

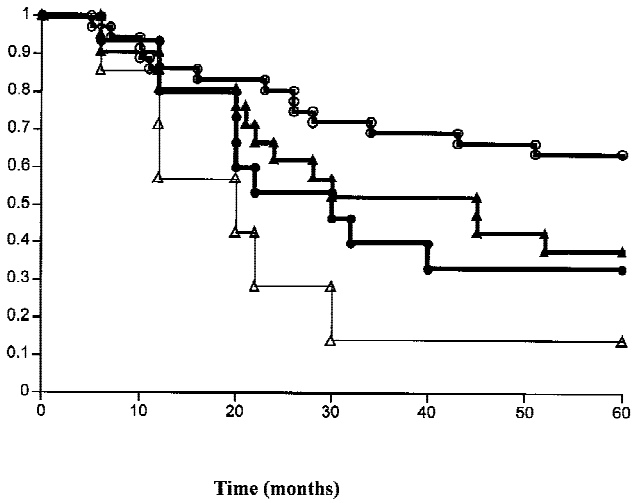

Fig. 1. Kaplan-Meier survival curves demonstrating that survival is reduced in patients whose tumor expressed PR (solid circles) versus patients whose tumor expressed neither ER nor PR (open circles)($P = 0.04$). In patients whose tumor expressed ER (solid triangles), survival was not significantly truncated. In patients whose tumor expressed both ER and PR (open triangles), survival was more markedly reduced ($P = 0.003$).

survival rate of 41.5% vs. a 5-year survival rate of 64% in patients whose tumor did not express ER or PR (NS). Similarly, patients with ER positive tumors had a 5-year survival of 38% which was not significantly different compared with receptor negative patients. Given the sample size, an alpha level of 0.05, and a 16-month period of accrual, the power of this comparison was 0.675. In patients whose tumor expressed PR, the 5-year survival rate was 33.3% and the median survival was 30 months, which was significantly shorter than the survival of patients with receptor negative tumors ($P = 0.04$; Fig. 1). Similarly, the 5-year survival rate of patients whose tumor expressed both ER and PR was 14.5% and the median survival was 20 months, which was significantly less than those patients with receptor negative tumors ($P = 0.003$).

Only the disease stage was shown to be an independent prognostic factor in a multivariate analysis model. Sex, age, tumor differentiation, and tumor location did not appear to have a significant influence on the long-term survival.

DISCUSSION

We postulated that expression of sex steroid receptors by colorectal adenocarcinoma was predictive of biological behavior. To test this hypothesis, we examined specimens of colorectal adenocarcinoma for the presence of ER and PR, correlating this with clinicopathologic characteristics and long-term outcome. We have demonstrated that advanced stage tumors express receptors more often than early stage tumors and that the presence of PR portends a worse survival.

Investigation of the potential role of sex steroid hormones in colorectal cancer was triggered by several clinical observations. In women, colorectal cancer is associated with cancers of the breast, uterus, and ovary [21,22]. In addition, early age at first pregnancy appears to protect against adenocarcinomas of the breast and colon [23]. On the other hand, several reports suggest that the use of hormone replacement therapy in postmenopausal women is associated with a decreased incidence of colon cancer [24,25]. Thus, epidemiologic studies suggest a role for sex steroid receptors in the development of colorectal cancer, but not all data are congruent.

Preclinical data also support a potential role of steroid hormones in carcinogenesis and tumor progression. Estradiol stimulates growth of gastric and colorectal cancer cell lines [26]. In Caco-2 cell lines, the mechanism for this proliferative response appears to be through stimulation of tyrosine and serine/threonine kinases. This response is abolished when genistein, a specific tyrosine kinase inhibitor, is added to the media [27]. Sex steroid receptors have been detected in high concentrations in dimethylhydrazine (DMH)-induced colon cancer in rats [28] and estradiol stimulates growth of experimentally induced colonic tumors in mice [29]. Furthermore, tamoxifen reduces the incidence of DMH-induced colon cancer in cell lines in vivo [30,31]. In contrast, earlier studies demonstrated that estrogen [32] and testosterone [33] have an inhibitory effect on receptor positive colorectal adenocarcinoma xenografts. Thus, most but not all experimental models of colonic adenocarcinoma suggest a trophic role for sex steroid hormones.

Studies evaluating expression of sex steroid receptors by normal and malignant colonic mucosa vary in their observations, most likely because of variations in methodology. In earlier studies using the Dextran-coated charcoal (DCC) assay, high levels of ER and PR proteins in

the cytosolic and nuclear fractions of both normal and malignant mucosa were seen [9–11,15,16]. In colonic polyps and malignant lesions, the degree of dysplasia was associated with the degree of expression of cytosolic and nuclear receptors [34]. Later studies testing fresh and paraffinized specimens by using a more specific monoclonal antibody-based immunoassay demonstrated low levels of ER and PR [35–37]. Finally, others evaluating ER and PR as markers in metastatic adenocarcinomas of unknown primary site failed to detect receptors in the malignant cells [38,39]. In our study, optimization of antigen retrieval by heat denaturation allowed the demonstration of nuclear receptors in 45% of specimens tested.

Preliminary results of another study evaluating cytosolic ER as a prognostic factor in colorectal adenocarcinoma suggest that the presence of receptors in normal mucosa surrounding the tumor is associated with longer survival [40]. In the same study, the presence of ER in the neoplastic tissue was of no prognostic value. In contrast, we found that expression of nuclear receptors for sex steroids, especially PR, correlated with a worse prognosis. In a number of specimens, receptors were also detected in the surrounding normal mucosa, although we could not perform a complete evaluation of the normal mucosa in all specimens, due to insufficient normal tissue.

Although most previous studies agree on the presence of sex steroid receptors in colorectal adenocarcinoma and normal colonic mucosa, there is controversy on which cell type expresses these receptors. Recent studies by others using in situ hybridization techniques and reversed transcriptase-polymerase chain reaction (RT-PCR) suggest that the cells expressing ER and PR are not of epithelial, but only of stromal origin [41,42]. In the present study, however, using immunohistochemistry, we identified ER and PR only in malignant epithelial cells.

The potential prognostic significance of receptors for sex steroids in other gastrointestinal tumors has been addressed by others. In patients with gastric adenocarcinoma, expression of ER and PR by the tumor has been shown to correlate with a worse survival [13]. Schirrous tumors expressing ER have a worse prognosis than ER negative tumors of similar morphology [17]. Furthermore, immunoreactivity to D5 antibody, which is directed to a nuclear ER, has been shown to be a negative prognostic factor in a large series of patients with gastric cancer [43]. As a therapeutic correlate, earlier studies from Japan showed a survival benefit for patients with gastric cancer treated with adjuvant chemotherapy and tamoxifen [44,45]. On the other hand, more recent studies using tamoxifen alone as adjuvant therapy versus no treatment have failed to reproduce these results [46]. The prognostic significance of ER/PR expression and the role of tamoxifen in the management of patients with gastric cancer therefore remains to be defined.

In the specimens evaluated in the present study, expression of ER and PR was associated with advanced stage disease. For this reason, in the multivariate analysis model, which included the stage, PR was not shown to be an independent prognostic factor. No significant difference in survival was demonstrated in patients whose tumor expressed ER alone and patients who had receptor negative tumors. However, the relatively low level of statistical power in this comparison suggests that examination of a larger study group might better demonstrate the prognostic effect of this marker.

Interestingly, the presence of PR in addition to ER was associated with an even more pronounced reduction in survival. It is unclear why the combined presence of PR and ER correlated with a particularly poor outcome. The observation that estradiol plays a regulatory role in synthesis of PR mRNA [47,48] suggests that expression of PR may potentiate function of ER and increase responsiveness to steroid hormones. This is consistent with our clinical findings.

CONCLUSIONS

By using an immunohistochemical assay, we confirmed the presence of ER and PR in the malignant mucosa of colorectal cancer specimens. We also demonstrated that ER and PR appear more frequently in later stages of disease. Further, the presence of PR or both receptors in the nuclei of malignant cells is associated with a truncated survival. Although our findings suggest that sex steroid hormones may play a role in the biological behavior of colorectal adenocarcinomas by promoting carcinogenesis and facilitating tumor growth, the clinical importance of this observation is yet to be defined. Expression of ER and PR suggests an important role of estrogen or progesterone in tumor progression and patient survival. Futher investigation with more patients studied prospectively is needed. Of particular importance is the definition of the potential role of pharmacological blockade of ER and PR which may prevent tumor progression and spread of disease.

ACKNOWLEDGMENTS

Special thanks to Efi Kokotou, MD, for her assistance in specimen processing and receptor analysis, and to Kara L. Hamilton, MPH, for her contribution to the statistical analysis.

REFERENCES

1. Ruder AM, Lubin F, Wax Y, et al.: Estrogen and progesterone receptors in breast cancer patients. Cancer 1989;64:196–202.
2. Gelber RD, Goldhirsch A, Coates AS: Adjuvant therapy for breast cancer: Understanding the overview. J Clin Oncol 1993;11:580–585.
3. Henderson BE, Ross RI, Bernstein L: Estrogens as a cause of

human cancer: The Richard and Hinda Rosenthal Foundation award lecture. Cancer Res 1988;48:246–253.
4. Crawford ED, Eisemberger MA, McLeod DG, et al.: A controlled trial of leuprolide with and without flutamide in prostatic carcinoma. N Engl J Med 1989;321:419–424.
5. Newcomb PA, Storer BE: Postmenopausal hormone use and risk of large-bowel cancer. J Natl Cancer Inst 1995;87:1067–1071.
6. Persson I, Yuen J, Bergkvist L, et al.: Cancer incidence and mortality in women receiving estrogen and estrogen-progestin replacement therapy: Long term follow-up of a Swedish cohort. Int J Cancer 1996;67:327–332.
7. Draser BS, Irving D: Enviromental factors and cancer of the colon and breast. Br J Cancer 1973;27:167–172.
8. Herrman JB: Breast cancer and associated extramammary malignant neoplasms. Am J Surg 1972;124:620–624.
9. Francavilla A, Di Leo A, Polimeno L, et al.: Nuclear and cytosolic estrogen receptors in human colon carcinoma and in surrounding noncancerous colonic tissue. Gastroenterology 1987;93:1301–1306.
10. Meggouh F, Lointier P, Pezet D, et al.: Status of sex steroid hormone receptors in large bowel cancer. Cancer 1991;67:1964–1970.
11. Meggouh F, Lointier P, Saez S: Sex steroid and 1,25-dihydroxyvitamin D3 receptors in human colorectal adenocarcinoma and normal mucosa. Cancer Res 1991;51:1227–1233.
12. Utsumi Y, Nakamura T, Nagasue N, et al.: Role of estrogen receptors in the growth of human esophageal carcinoma. Cancer 1989;64:88–93.
13. Matsui M, Kojima O, UeharaY, et al.: Characterization of estrogen receptor in human gastric cancer. Cancer 1991;68:305–308.
14. Greenway B, Oqbal MJ, Johnson PJ, et al.: Oestrogen receptor protein in malignant fetal pancreas. Br Med J 1989;283:751–753
15. Stedman KE, Moore GE, Morgan RT: Estrogen receptor proteins in diverse human tumors. Arch Surg 1980;115:244–248.
16. Alford TC, Do HM, Geelhoed GW, et al.: Steroid colon receptors in human colon cancers. Cancer 1979;43:980–984.
17. Yokozaki H, Takekura N, Takanashi A, et al.: Estrogen receptors in gastric adenocarcinoma: A retrospective immunohistochemical analysis. Virchows Arch [A] 1988;413:297–302.
18. Kojima O, Takahashi T, Kawakami S, et al.: Localization of estrogen receptors in gastric cancer using immunohistochemical staining of monoclonal antibody. Cancer 1991;67:2401–2406.
19. American Joint Committee on Cancer: Colon and rectum. In Fleming ID, Cooper JS, Henson DE, et al. (eds): "AJCC Cancer Staging Manual" (5th ed). Philadelphia: Lippincott-Raven, 1998: 83–87.
20. Norton AJ, Jordan S, Yeomans P: Brief, high temperature heat denaturation (pressure cooking): A simple and effective method of antigen retrieval for routinely processed tissues. J Pathol 1994; 173:371–379.
21. Fraumeni JF Jr, Lloyd JW, Smith EM, et al.: Cancer mortality among nuns: Role of marital status in etiology of neoplastic disease in women. J Natl Cancer Inst 1969;42:455–468.
22. Choenberg BS, Greenberg RA, Eisenberg H: Occurrence of certain multiple primary cancers in females. J Natl Cancer Inst 1969; 43:15–32.
23. Weiss NS, Daling JR, Chow WH: Incidence of cancer of the large bowel in women in relation to reproductive and hormonal factors. J Natl Cancer Inst 1981;67:57–60.
24. Troisi R, Schairer C, Chow WH, et al.: A prospective study of menopausal hormones and risk of colorectal cancer (United States). Cancer Caus Contr 1997;8:130–138.
25. Kampman E, Potter JD, Slattery ML, et al.: Hormone replacement therapy, reproductive history, and colon cancer: A multicenter case-control study in the United States. Cancer Caus Contr 1997; 8:146–158.
26. Harrison JD, Watson S, Morris DL: The effect of sex hormones and tamoxifen on the growth of human gastric and colorectal cancer cell lines. Cancer 1989;11:2148–2151.
27. Di Domenico M, Castoria G, Bilancio A, et al.: Estradiol activation of human colon carcinoma-derived Caco-2 cell growth. Cancer Res 1996;56:4516–4521.
28. Odagiri E, Jibiki K, Kato Y, et al.: Steroid receptors in dimethylhydrazine-induced colon carcinogenesis. Cancer 1985;56:2627–2634.
29. Narayan S, Rajakumar G, Prouix H, et al.: Estradiol is trophic for colon cancer in mice: Effect on ornithine decarboxylase and c-myc messenger RNA. Gastroenterology 1992;103:1823–1832.
30. Ziv Y, Fazio VW, Gupta MK, et al.: Effect of tamoxifen on 1,2-dimethylhydrazine-HCL-induced colon carcinogenesis in rats. Anticancer Res 1997;17:803–810.
31. Di Leo A, Linsalata M, Cavallini A, et al.: Sex steroid hormone receptors, epidermal growth factor receptors and polyamines in human colorectal cancer. Dis Colon Rectum 1992;35:305–309.
32. Izbicki JR, Scmitz R, Kamran D, et al.: Androgens as promoters of colon carcinogenesis. Cancer Detect Prev 1983;6:355–362.
33. Stebbings WS, Vinson GP, Farthing MJ, et al.: Effect of steroid hormones on human colorectal adenocarcinoma xenografts, of known steroid-receptor status, in nude mice. J Cancer Res Clin Oncol 1989;115:439–444.
34. Concolino G, Buonomo O, Conti C, et al.: Nuclear steroid receptors and dysplasia in adenomatous polyps of the colon as markers of high risk of malignant transformation. Cancer Detect Prev 1986;9:477–484.
35. Galandiuk S, Miseljic S, Yang AR, et al.: Expression of hormone receptors, cathepsin D, and HER-2/neu oncoprotein in normal colon and colonic disease. Arch Surg 1993;128:637–642.
36. Hendrickse CW, Jones CE, Donovan IA, et al.: Oestrogen and progesterone receptors in colorectal cancer and human colonic cancer cell lines. Br J Surg 1993;80:636–640.
37. Cameron BL, Butler JA, Rutgers J, et al.: Immunohistochemical determination of the estrogen receptor content of gastrointestinal adenocarcinomas. Am Surg 1992;58:758–760.
38. Ollayos CW, Riordan GP, Rushin JM: Estrogen receptor detection in paraffin sections of adenocarcinoma of the colon, pancreas and lung. Arch Pathol Lab Med 1994;118:630–632.
39. Brown RW, Campagna LB, Dunn JK, et al.: Immunohistochemical identification of tumor markers in metastatic adenocarcinoma. Am J Clin Pathol 1997;107:12–19.
40. Di Leo A, Messa C, Russo F, et al.: Prognostic value of cytosolic estrogen receptors in human colorectal carcinoma and surrounding mucosa. Preliminary results. Digest Dis Sci 1994;39:2038–2042.
41. Singh S, Poulsom R, Hamby AM, et al.: Expression of oestrogen receptor and oestrogen-inducible genes pS2 and ERD5 in large bowel mucosa and cancer. J Pathol 1998;184:153–160.
42. Waliszewski P, Blaszczyk M, Witort EW, et al.: Molecular study of sex steroid receptor gene expression in human colon and in calorectal carcinomas. J Surg Oncol 1997;64:3–11.
43. Harrison JD, Jones JA, Ellis IO, et al.: Oestrogen receptor D5 antibody is an independent negative prognostic factor in gastric cancer. Br J Surg 1991;78:334–336.
44. Kitaoka H: Sex hormone dependency and endocrine therapy for patients with diffuse carcinoma of the stomach. Jpn J Cancer Chemother 1983;10:2453–2460.
45. Kojima O, Takahashi T: Endocrine therapy for scirrhous carcinoma of the stomach. Jpn J Cancer Chemother 1986;13:2526–2531.
46. Harrison JD, Morris DL, Ellis IO, et al.: The effect of tamoxifen and estrogen receptor status on survival in gastric carcinoma. Cancer 1989;64:1007–1010.
47. Nardulli AM, Greene GL, O'Malley BW, et al.: Regulation of progesterone receptor messenger ribonucleic acid and protein levels in MCF-7 cells by estradiol: Analysis of estrogen's effect on progesterone receptor synthesis and degradation. Endocrinology 1988;122:935–944.
48. Ing NH, Tornesi MB: Estradiol up-regulates estrogen receptor and progesterone receptor gene expression in specific ovine uterine cells. Biol Reprod 1997;56:1205–1215.